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Published in:
Biochimica et Biophysica Acta (BBA)

DOI:
10.1016/j.bbadis.2019.01.022

Publication date:
2019

Document Version
Author accepted manuscript

Knockout of Nr2e3 prevents rod photoreceptor differentiation and leads to selective L-/M-cone photoreceptor degeneration in zebrafish

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Abstract

Mutations in the photoreceptor cell-specific nuclear receptor gene Nr2e3 increased the number of S-cone photoreceptors in human and murine retinas and led to retinal degeneration that involved photoreceptor and nonphotoreceptor cells. The mechanisms underlying these complex phenotypes remain unclear. In the hope of understanding the precise role of Nr2e3 in photoreceptor cell fate determination and differentiation, we generated a line of Nr2e3 knockout zebrafish using CRISPR technology. In these Nr2e3-null animals, rod precursors undergo terminal mitoses but fail to differentiate as rods. Rod-specific genes are not expressed and the outer segment (OS) fails to form. Formation and differentiation of cone photoreceptors is normal. Specifically, there is no increase in the number of UV-cone or S-cone photoreceptors. Laminated retinal structure is maintained. After normal development, L-/M-cones selectively degenerate, with progressive shortening of OS that starts at age 1 month. The amount of cone phototransduction proteins is concomitantly reduced, whereas UV- and S-cones have normal OS lengths even at age 10 months. In vitro studies show Nr2e3 synergizes with Crx and Nrl to enhance rhodopsin gene expression. Nr2e3 does not affect cone opsin expression. Our results extend the knowledge of Nr2e3's roles and have specific implications for the interpretation of the phenotypes observed in human and murine retinas. Furthermore, our model may offer new opportunities in finding treatments for enhanced S-cone syndrome (ESCS) and other retinal degenerative diseases.

Keywords Nr2e3, CRISPR, Zebrafish, Photoreceptor, Differentiation, Degeneration

1. Introduction

To cope with the enormous range of ambient light levels encountered in daily life, the vertebrate retina utilizes two types of photoreceptors: rods that function in dim light and cones that function in bright light and mediate color vision. Although the signaling pathway for phototransduction is common for rods and cones, the signalling proteins are mostly coded by distinct sets of rod and cone specific genes. The human retina has one type of rod and three types of cone. Rods express the visual pigment rhodopsin, whereas the three types of cones express the short (S, blue), medium (M, green) or long (L, red) wavelength cone opsin. The murine retina has one type of rod and only S- (blue) and M- (green) cones; some cones express both S and M opsins. The zebrafish has one type of rod and four types of cone: L-, M-, S- and UV-cone. The fourth type expresses a cone opsin that is maximally sensitive to UV light [1–6]. During development, the rods, cones and five other major classes of retinal cells are produced from a common pool of pluripotent progenitor cells. The progenitors undergo mitosis and the post-mitotic cells then undergo differentiation into specific types of retinal cells. Differentiation is conceptualized as a process of selective expression of sets of specific genes [7–12]. For rods and cones, several transcription factors that control photoreceptor-specific gene expression have been identified. Cone-rod homeobox transcription factor (Crx) is central to photoreceptor determination [13–15] and required for the development of photoreceptor outer segment (OS) and expression of photoreceptor-specific genes [14–17]. Crx is expressed both in rod and cone photoreceptors. Mutations in Crx cause photoreceptor degeneration in humans (autosomal dominant cone-rod dystrophy (CRD) [13,18], autosomal dominant retinitis pigmentosa (adRP) [19] and autosomal recessive Leber congenital amaurosis (LCA) [20]) and in mice [14]. Neural retina leucine zipper protein (Nrl) is a leucine zipper transcription factor that is required for rod development. Nrl is preferentially expressed in rods. It acts synergistically with Crx to regulate rhodopsin transcription. Mutations in human Nrl cause retinal degeneration: autosomal dominant retinitis pigmentosa (adRP) [21], autosomal recessive retinitis pigmentosa (arRP) [22] and

enhanced Scone syndrome (ESCS) [23]. More details about ESCS and RP will be presented later. Deletion of Nrl in mice caused the complete loss of rod function and super-normal S-cone function. In the absence of Nrl, the rods acquired some of the characteristics of S-cones. Accordingly, Nrl seems to act as a molecular switch during rod-cell development by directly modulating rod-specific genes while simultaneously inhibiting the S-cone pathway through the activation of Nr2e3 [24].

The nuclear receptor transcription factor Nr2e3 is expressed in photoreceptors. It forms a complex with Crx that enhances the expression of rod-specific genes and represses the expression of cone specific genes in rods [25,26]. Mutations in human Nr2e3 cause ESCS, an autosomal recessive disorder characterized by reduced rod, L- and M-cone responses and enhanced sensitivity to short-wave light due to supernumerary S-cones. In addition, patients with ESCS have dysmorphic retinas, showing an early disorganization of the laminated retinal structure with rosette formation in the outer nuclear layer (ONL) where photoreceptor cell bodies are located, and a slow, progressive retinal degeneration [27–30]. Mutations in Nr2e3 have also been identified in patients with Goldmann-Favre syndrome, clumped pigmentary retinal degeneration [31], adRP [32], and arRP [33]. A spontaneous recessive null mutation in the murine Nr2e3 has been identified in the retinal degeneration 7 (rd7) mice. The phenotype of this mutant includes progressive photoreceptor degeneration and attenuation of the electroretinogram (ERG) with time, abnormal retinal lamination with rosette formation in the ONL and a 2-fold increase in the number of S-cones [34,35]; however, the majority of the photoreceptors in the rd7 retina are a hybrid cell type that expresses both rod and cone-specific genes [36].

The roles played by Nrl and Nr2e3 in rod/cone cell fate determination and differentiation have been exploited in a strategy of therapeutic cellular reprogramming to treat RP, a retinal degeneration characterized by primary rod defects and degeneration followed by secondary cone loss. Many RP-causing mutations occur in rod-specific genes. The rationale for such a strategy is that knocking down rod determinants such as Nrl or Nr2e3 reprograms rods to a cone fate, rendering

rod-specific gene mutations irrelevant, with consequent preservation of retinal structural integrity and function [37–40].

Although much is known about the function of Nr2e3, major questions remain unanswered. For example, the role of Nr2e3 in retinal progenitors for cell fate determination is unclear [5]. In Nr2e3$^{-/-}$ retinas, the aberrant S-cones could have arisen from an alteration of cell fate or dedifferentiation of postmitotic premature rods [25].

Given the strategic importance of these transcription factors in potential therapeutic intervention, it is important to better understand their function. We have decided to study Nr2e3 in zebrafish by knocking out the gene using clustered regularly interspaced short palindromic repeat (CRISPR) technology. Architecture and cell types in zebrafish retina are conserved with that in human; furthermore, the zebrafish is diurnal and possesses a cone dominant retina similar to the macula in human retina [41–43]. Importantly, genomic structure and amino acid sequence of zebrafish Nr2e3, especially the DNA-binding domain (DBD) and ligand binding domain (LBD), is highly conserved with the human ortholog [25,44]. For these reasons, zebrafish could be a suitable organism to study the roles of Nr2e3 underlying photoreceptor development and differentiation. Here, we report on findings from the study of such Nr2e3-null zebrafish.

2. Materials and methods

2.1. Zebrafish maintenance and breeding

Wild-type AB strain of zebrafish were raised in recirculating water system (pH 6.6–7.4, 26–28.5 °C) and fed with brine shrimps three times a day under the condition of 14/10 h light/dark cycle. Embryos were kept in E3 medium (5mM NaCl, 0.17mM KCl, 0.33mM CaCl2, 0.33mM MgSO4) at 30 °C. The medium was changed once a day, after 96 h incubation, the larvae were transferred into tanks and fed with live paramecia three times a day. Our study was approved by the Ethics Committee of Huazhong University of Science and Technology.

2.2. Cas9 mRNA and gRNA synthesization

Plasmids that contain the template DNA for in vitro transcription of Cas9 mRNA were linearized by NotI and purified by ethanol precipitation. Capped Cas9 mRNA was synthesized using the mMESSAGE Mmachine SP6 kit (Ambion, Austin, Texas, USA) and purified by LiCl precipitation. Plasmids that contain the gRNA scaffold sequence were linearized by BamHI and purified by ethanol precipitation. gRNA was synthesized using TranscriptAid T7 High Yield Transcription Kit (Thermo scientific, Wilmington, Delaware, USA) and purified by phenol: chloroform extraction and isopropanol precipitation.

2.3. Microinjection and genotyping

The compounds of gRNA and Cas9 mRNA were injected into one cell stage WT embryos according to previously reported protocol [45]. Effective groups were raised to sexual maturity and outcrossed with WT fish to obtain F1 zebrafish. F1 males and F1 females carrying the same mutations were crossed; homozygous fish were generated. A pair of primers (5′-TAGTTTCTGCCCTTATTGTCTT-3′, 5′-CTTGTCTGATTGGAATGTTGTT-3′) that flanked the target site was used to amplify the 480 bp DNA fragments; these amplifying fragments were then identified by agarose electrophoresis and DNA sequencing. The homozygotes were also confirmed by quantitative Real-time PCR and Western blot.

2.4. Hematoxylin and eosin (H&E) staining

Whole mount zebrafish larvae or isolated eyes were fixed in the 4% paraformaldehyde (PFA) overnight at 4 °C. Next, larvae or eyes were dehydrated in 30% sucrose (diluted in PBS). After that, the tissues were embedded in OCT compound; embedded tissues were positioned parallel to the blade and were sectioned sagittally. For larvae each slice was 8 μm thick, and for eyes each slice was 12 μm thick. Operations were followed as in previous protocol [46,47]. We chose the slices containing optic nerve and stained with hematoxylin and eosin. Images were captured by Olympus BX53 microscope (Olympus Imaging, Tokyo, Japan).

2.5. Immunofluorescence

Retinal cryosections from WT and Nr2e3$^{-/-}$ zebrafish were put at room temperature for 20 min for drying. Then the sections were permeabilized in PDT (PBS containing 1% DMSO and 0.1% TritonX-100) for 10 min. After being blocked with 10% goat serum in PBDT (PDT containing 1% BSA) for 1 h, tissues on the slide were incubated with primary antibody overnight at 4 °C. The primary antibodies used in this study and the dilution ratio are shown in Supplementary Table 1. For the next step, slides were washed in PDT 3 times and then incubated with Alexa Fluor 488 nm secondary antibody (Life Technologies, Carlsbad, California, USA) at 37 °C for 1 h. DAPI (Sigma, St. Louis, Missouri, USA) was diluted with PBS and used to label nuclei at a concentration of 5 μg/ml. Images were acquired using a confocal laser scanning microscope (FluoView FV1000 confocal microscope, Olympus Imaging). All operations were followed as previous protocol [48,49].

2.6. Transmission electron microscopy

Adult zebrafish eyes or whole body of larval zebrafish were fixed in 2.5% glutaraldehyde in 0.1M PBS. After washing 3 times, the eyes were further fixed in 1% osmium tetroxide in 0.1M PBS buffer at room temperature for 2 h. For the next step, the eyes were dehydrated in gradient ethanol and then incubated in acetone for 20 min at room temperature. The eyes were treated with 50% (1 h), 75% (3 h) and 100% (overnight) epoxy resin (mixed with acetone, v/v) successively, and then heated at 70 °C overnight. Ultrathin sections were produced using a Reichert-Jung ultramicrotome (Leica, Wetzlar, Germany). Sections were stained with 3% uranyl acetate and 3% lead citrate. Transmission electron microscopy (HT7700, Hitachi, Tokyo, Japan) was used to obtain images.

2.7. RT-PCR and Western blotting

Total RNAs were extracted from embryos or larvae using RNAiso Plus reagent (Takara, Kusatsu, Japan), cDNA was generated by MMLV reverse transcriptase (Invitrogen, Carlsbad, California, USA). The cDNA was used as templates to examine the mRNA level of targeted genes. These experiments were performed using AceQ™ qPCR SYBR® Green Master Mix (Vazyme, Nanjing, China) on the

StepOnePlus™ real-time PCR System (ABI, Carlsbad, California, USA). The primers used for specific gene are listed in Supplementary Table 2. Proteins were extracted from adult fish eyes and homogenized in RIPA buffer (Beyotime, Shanghai, China) with protease inhibitor cocktail (Roche, Basel, Switzerland). Concentrations of proteins were determined using the BCA Protein Assay Kit (Beyotime). Lysates were mixed with loading buffer and heated in boiling water for 10 min, then stored immediately at −20 °C. The same amounts of protein were separated on SDS–PAGE and transferred to nitrocellulose membranes (Millipore, Danvers, Minnesota, USA). The membranes were then orderly blocked and incubated in primary antibodies overnight. Antibodies used for WB are listed in Supplementary Table 1. The membranes were washed and incubated in HRP-conjugated secondary antibodies (Thermo Fisher). Finally, the membranes were visualized using ChemiDoc™ XRS +system (Bio-Rad Laboratories, Berkeley, California, USA) to acquire the images. All operations were followed as in previous protocol [50–52].

2.8. EdU and TUNEL assays

A Cell-light EdU Apollo567 in Vitro Kit (RiboBio, Guangzhou, China) and a BrightRed Apoptosis Detection Kit (Vazyme) were used to detect the proliferous and apoptotic cells according to the manufacturer's instructions. Images were captured using a confocal microscope (FV1000, Olympus).

2.9. Cell culture, plasmids and transfection

Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, New York, USA) containing 10% fetal bovine serum (FBS, Gibco) was used to raise HEK293 cells. The full length of zebrafish Nr2e3 coding sequence was cloned into the p3XFLAG-CMV-7.1 vector; the full length of zebrafish Crx and Nrl coding sequences were cloned into pEGFP-C1 vector. Cell-type specific promoter sequences were amplified using specific primers and cloned into the pGL3 Luciferase Reporter Vectors. Primers used for vector construction are listed in Supplementary Table 3. Plasmids were transfected into HEK293 cells with Lipofectamine 3000 Reagent (Thermo Fisher) in Opti-MEMI Reduced Serum

Medium (Gibco). After 6 hour incubation, the medium was changed with DMEM containing FBS; after 36 hour incubation, cells were harvested and activity of luciferase was examined using Dual-Luciferase® Reporter Assay System (Promega, Madison, Wisconsin, USA).

2.10. Statistical analysis

All experiments were repeated at least three times. Significance was determined by two-tailed Student's t-test.

3. Results

3.1. Generation of Nr2e3 mutant zebrafish strain by CRISPR/Cas9 technology

In order to generate the Nr2e3 knockout zebrafish strain, cDNA (NM_001007368.1) sequence was screened on the special website (http://www.crisprscan.org/) for a high score oligo that was suitable to design as a target site: a 23-bp sequence located in exon 4 was thus selected (Fig. 1A). Cas9 mRNA and gRNA transcribed in vitro were microinjected into one-cell-stage embryos and mutagenic efficiency was evaluated after 48 hpf. All procedures followed previous protocol [45]. We identified a mutation that carries a 37 bp deletion c.485_521del in Nr2e3 mRNA. This deletion caused a frame shift resulting in an abnormal p.Leu162Glnfs*30 Nr2e3 protein (Fig. 1B). The mRNA level of Nr2e3 was reduced to nearly 40% compared with those of WT control, an effect probably explained by nonsense-mediated RNA decay (Fig. 1C). Western blot assay was performed to evaluate the protein level of Nr2e3. A 46 kD protein was detected in WT extracts but was lost in the mutant samples (Fig. 1D). Thus, our knockout zebrafish are authentic Nr2e3-null mutants. The mutant animals appeared smaller during the larval period, but there was no difference in body size at juvenile and adult stages. No other macroscopic phenotypes were observed.

3.2. Differentiation of rod photoreceptors is arrested in Nr2e3-null zebrafish

Because Nr2e3 was reported to express primarily in rods [25], the phenotype of rods was first analyzed in Nr2e3 mutant zebrafish. Immunofluorescent staining was conducted using anti-

rhodopsin antibody to label rod OSs. At 10 dpf, rhodopsin immunolabelling was observed clearly at the ventral and dorsal regions in WT retinas. The labelled pattern of dense cells in ventral retina, sporadic cells in dorsal retina and few cells in central retina is consistent with the depiction of origination and gradient distribution of rods [53]. However, rhodopsin immunolabelling was not found in Nr2e3 mutant retinas (Fig. 2A). We examined this aspect of the phenotype in adult retinas at age 6 months and 10 months. Similarly, no rhodopsin labelled signals were detected in Nr2e3 mutant retinas (Fig. 2A). The mRNA levels of rod genes rhodopsin, gnat1, grk1a and pde6b were evaluated by the real-time quantification PCR method. Results showed that these genes were not expressed (Fig. 2B). Consistent with the mRNA results, protein levels of rhodopsin, GNAT1, PDE6B and GNB1 were not expressed (Fig. 2C). These data indicate that rods were not developed in Nr2e3 mutant zebrafish. However, expression of rod genes were rescued when Nr2e3 mRNA was injected into the mutant embryos, this suggested that the phenotypes of $Nr2e3^{-/-}$ zebrafish are due to loss of Nr2e3 (Fig. S1).

To verify the above observations directly, we performed transmission electron microscopic (TEM) analysis to examine the ultrastructure of the mutant retinas. At 10 dpf, unambiguous rod OSs developed well in WT retinas, but only cone OSs were found in Nr2e3 mutant retinas (Fig. 3A, B). At 1 month, by contrast to the regularly organized and well-formed rod OSs in WT fish, no rod OSs were found in Nr2e3 mutants (Fig. 3C–D').

Consistent with the lost expression of rod genes and lack of rod OSs, the number of rod nuclei was reduced. Based on the locations and morphological features of rod and cone nuclei [36], we identified small and round rod nuclei arranged regularly in WT ONL, but only sporadic rod nuclei were found in Nr2e3 mutant retinas (Fig. S2). This feature could be seen clearly by DAPI staining (Fig. 2A).

Nr2e3 has been reported to express in rod progenitors and precursors in zebrafish [53]. Thus, we wondered if generation of rod lineage was implicated. The mRNA level of pax6, a marker of Müller

glial cells that were identified as rod progenitors [53,54], was not changed (Fig. 4A). The mitotic cells in the central retina that possessed a rounded or fusiform shape were considered as rod progenitors or precursors [54,55]. Therefore, EdU incorporation assays were performed on our zebrafish. Consistent with the previous report, most EdU labelled cells were located at the dorsal and ventral ciliary marginal zone (CMZ), the persistent proliferative regions in the zebrafish retina [53,56]. We identified EdU positive cells that possessed a rounded or fusiform shape in the central ONL or INL of the mutant (arrowheads in Fig. 4B). Furthermore, the labelled pattern of zpr-3, another rod marker, at 72 hpf when rod lineage had emerged but before rhodopsin could be detected, was identical between WT and Nr2e3 mutant retinas (Fig. 4C). These results show that generation of rod lineage is not affected.

To determine if these immature rods would undergo apoptosis before expressing the mature markers and developing OSs, we examined the retinal sections from WT and Nr2e3$^{-/-}$ mutants at 10 dpf using terminal deoxynucleotidyl transferase dUTP nickend labeling method (TUNEL). In 18 WT retinal sections we detected 3 TUNEL positive cells in ONL and 12 cells in INL or GCL; by contrast, in 18 mutant sections no labelled cells were found in ONL and only 6 positive cells in INL or GCL (Fig. S3). So, the loss of rods in Nr2e3 mutants could not be explained by apoptosis.

In conclusion, these results indicate that Nr2e3 is required for rod differentiation but does not act in rod fate determination.

3.3. Cell fate determination and differentiation of cone photoreceptors are not affected in Nr2e3 mutant zebrafish

Since in ESCS patients and rd7 mice, Nr2e3 mutations caused an overproduction of S-cones, thus we wondered if the immature rods were transformed into UV-cones, the closest homolog of mammalian S cones [43]. Anti-SWS1 antibody was used to immunolabel the UV-cone OSs. Unlike the phenotypes in ESCS and rd7, UV-cones did not increase (Fig. 5A). We then examined the number of S-cones using anti-SWS2 antibody. Similarly, S-cones were not increased (Fig. 5B). The mRNA levels

of cone genes opn1lws1, opn1lws2, pde6c and a transcription factor tbx2b, which is required for fate determination of UV-cones [10], were not changed (Fig. S4A). The observation that rods were lost but UV- and S-cones did not increase prompted us to analyze the phenotype of L-/M-cones. Anti-LWS and anti-RH2 antibodies were used to immunolabel L-/M-cone OSs, respectively. Again, the number of L-/M cones was not altered (Fig. 5C, D). Consistent with these results, the labelled patterns of PNA (peanut lectin) and GNAT2 (cone transducing subunit), proteins that are located in cone OSs, were identical to those in WT sections (Fig. S4B, C). These results suggest that formation and differentiation of cone photoreceptors proceed as normal in the mutant zebrafish. Specifically, there is no increase in the number of S-cone or UV-cone photoreceptors.

A previous study showed that loss of Nr2e3 resulted in superabundant proliferation of cone progenitors in rd7 mouse [5]. Therefore, we counted the number of EdU positive cells at the peripheral ONL, which are considered as nascent cone precursors [57], and found no difference between WT and mutant (arrows in Figs. 4B, S5). These data suggest that proliferation of cone progenitors does not increase in Nr2e3 mutant zebrafish.

3.4. Laminated retinal structure is maintained in Nr2e3 mutant zebrafish

Histological examinations were implemented to analyze the whole structure of mutant retinas. Retinal cryosections from WT and mutants at different ages were stained with hematoxylin and eosin. The laminated architecture was maintained in Nr2e3 mutant retinas. Unlike the phenotype of rd7 mouse [34], there were no whorls or rosettes in the ONL. Notably, from 1 month of age the lengths of photoreceptor OSs were gradually reduced. Consider that there were no rod OSs in mutant retinas, we surmised that cone OSs were shortened. At all stages that we analyzed, the thickness of the inner retina was normal (Figs. 6A–D, S6).

3.5. Selective degeneration of L-/M-cone photoreceptors was observed in Nr2e3 mutant zebrafish

Our histological data indicated that degeneration of cone photoreceptors was occurring in Nr2e3 mutant zebrafish. To determine which cone subtypes were involved, immunostaining assays were implemented to analyze the phenotypes of the four types of cones in several adult stages. We found that the lengths of UV-cone and S-cone photoreceptor OSs were normal at 4 months and 10 months but OSs of L-/M-cones were shortened gradually, starting at age 1 month (Figs. 7A–D, S7A–D). In 6 months old retinas that were immunolabelled using anti-GNAT2 antibody, the labelled OSs were observed to be dividing into three layers in WT retinas. According to their spatial orientation and shape, these were OSs of UV-cones, S-cones and L-/Mcones. Only the OSs of L-/M-cones were shortened significantly (Fig. S8A). Consistent with the shortened OSs, the protein levels of GNB3 and GNAT2 were reduced dramatically at 6 months and 12 months (Fig. S8B). These results suggest that L-/M-cones selectively degenerate in Nr2e3 mutant zebrafish.

3.6. Nr2e3 enhances transcription of rhodopsin synergized with Crx and Nrl, but does not repress that of UV-or S-cone opsins

It is widely accepted that Nr2e3 promotes rod differentiation by strengthening the expression of rod genes and repressing the expression of cone genes in rods. These opposing roles are achieved by cooperation with other transcription factors, especially Crx and Nrl [24–26,58]. From the phenotype of our mutant, which showed different effects the loss of Nr2e3 had on rods and cones, we uspected that zebrafish Nr2e3 would strengthen expression of rod genes synergized with Crx and Nrl, but would not affect that of cone genes. To confirm this hypothesis, reporter assays were conducted in HEK293 cells. The activity of rhodopsin promoter (2.5 kb) was examined. We showed that Nr2e3 alone did not increase rhodopsin promoter activity. Co-expressing Crx and Nrl increased the activity by 2.8-fold. When Nr2e3 was expressed at the maximum concentration that we have assayed, rhodopsin promoter activity was increased by 4-fold, which was significantly higher than that expressing Nr2e3 alone or co-expressing Crx and Nrl (Fig. 8A). This property of zebrafish Nr2e3 on regulation of rhodopsin expression is conserved with that in mammalian retina [26].

The activity of rhodopsin promoter in this study is different from previous reports in that the mammalian rhodopsin promoter can be activated 70- or 175-fold [26,59]. The length of the 2.5 kb promoter sequence ought to be sufficient because in RHO-EGFP zebrafish a 1 kb sequence of rhodopsin promoter could drive EGFP expression in rod photoreceptors. It is possible that there are enhancers far from the promoter, or there are unidentified factors playing a positive role for expression of rhodopsin that were not included in the complexes, so that the promoter did not reach the maximal activity.

To explain the phenotypes of UV- and S-cones, we tested the role of Nr2e3 on expression of UV and blue opsin genes. We found that Nr2e3 did not repress transcription of UV- or S-opsin (Fig. 8B, C). This property of zebrafish Nr2e3 is different from that of the mammalian ortholog [26]. Because zebrafish Nr2e3 did not repress expression of UV or S cone opsins, expression of these genes would not undergo expansion when Nr2e3 was lost, thus the number of UV or S cones would not increase in Nr2e3 mutant zebrafish.

Together, our reporter assays indicate that in zebrafish, Nr2e3 may promote rod differentiation by reinforcing expression of rod genes. The fact that Nr2e3 does not repress expression of UV or S cone genes may explain the phenotype of UV- and S-cones.

4. Discussion

In the hope of understanding the precise role of Nr2e3 and providing interpretation of the phenotypes of ESCS and rd7, an Nr2e3-null zebrafish model was created in this study. We show that Nr2e3 has no role in photoreceptor cell fate determination. It is necessary for rod but not cone photoreceptor differentiation. Lack of Nr2e3 leads to selective L-/M-cone photoreceptor degeneration.

Loss of Nr2e3 function leads to very different outcomes for rod development in mouse and in zebrafish. In the rd7 mouse, rod genes were expressed normally at the mRNA and protein levels [5,25,36,60]. The physiological function of rods was maintained initially [61]. These observations are

consistent with normal development of rods in the rd7 mouse. By contrast, the post-mitotic premature rods of Nr2e3$^{-/-}$ zebrafish fail to develop at all. The phenotype of Nr2e3$^{-/-}$ zebrafish resembles that of Nr$^{l-/-}$ mouse in that deletion of the gene ablated expression of rod genes and prevented formation of rod OSs. The physiological function of rods was not detected [24]. These data demonstrated that development of rods was prevented totally in Nrl−/− mouse. Accordingly, analogous to Nrl's role in the murine retina, zebrafish Nr2e3 acts like a “molecular switch” in modulating rod-specific genes. The critical role Nrl and Nr2e3 each play in rod development in, respectively, mouse and zebrafish, may be reflected in the developmental order of expression of these two genes. In mouse, Nrl transcripts appear at ~E12.5 [62] and before Nr2e3 at ~E16.5 [59], while in zebrafish the expression of Nr2e3 is earlier than Nrl in rod lineage. Nr2e3 appears first in rod progenitors in the INL and in all rod precursors in the ONL. Nrl transcripts appear only in the ONL [53]. Unlike the situation in mouse where Nrl can activate expression of Nr2e3 [24], the earlier appearance of Nr2e3 transcripts in zebrafish means that its expression is not activated by Nrl. ESCS patients present with severe deficit of rod function at an early stage and histological analysis of one postmortem retina revealed an absence of rods [26,27,57]. Furthermore, retinal structural analysis led to the conclusion that rods failed to differentiate in ESCS retinas [27]. During fetal development, Nr2e3 expression follows Nrl very closely, and mutations in either Nrl or Nr2e3 can lead to ESCS [63], suggesting these two genes may play parallel roles in rod development.

The phenotypes of ESCS, Nr2e3$^{-/-}$ zebrafish, Nrl$^{-/-}$ mouse and rd7 mouse demonstrate that Nr2e3 has a critical role for rod development in zebrafish and human. By contrast, it is Nrl that modulates rod development in mouse. However, Nrl alone is not sufficient to maintain rod survival in rd7 mouse. Therefore, Nr2e3 may play a role in rod maintenance.

There are two hypotheses that may account for the enhanced Scones in ESCS and rd7 mouse. One is that lack of Nr2e3 function leads the plastic rod precursors to transdifferentiate into S-cones. Two lines of evidence support this hypothesis: 1) Nr2e3 is predominantly expressed in developing

and mature rods and plays a dual role in promoting expression of rod genes and repressing cone genes [25]; 2) there are hybrid rod-cone photoreceptors expressing both rod and cone genes in the rd7 mouse [36]. The second hypothesis is that losing Nr2e3 causes aberrant proliferation of cone progenitors. Observations that Nr2e3 is expressed in cone progenitors and mature cones, as well as the fact that there are no hybrid photoreceptors, support this hypothesis [5]. In zebrafish, we observed Nr2e3 function only in rod lineage; cone development and proliferation of cone progenitors were not changed. Thus, our data contradict the second hypothesis. However, there are remaining questions about the first hypothesis. Rods account for 97% of the number of photoreceptors in the mouse retina, while the S-cones increased only 2-fold in the rd7 retina [34]. This suggests only a small fraction of rod precursors were transformed into S-cones. Because a fraction of rods were derived from the default S-cones in mouse [43], perhaps it was these rods that reverted back to S-cones when Nrl/Nr2e3 was lost. The enhanced S-cones were actually the default S-cones. By contrast, UV- or S-cone is not the default photoreceptor in zebrafish [10,43] (and the current data). Thus, the number of these two cone types is not increased when rods were lost in Nr2e3−/− zebrafish.

The mechanism that causes selective L-/M-cone photoreceptor degeneration remains unknown. In ESCS and rd7, arrangement of the ONL was disorganized and this disorganization was present before photoreceptor degeneration [35,64]. Disorganized arrangement of the ONL (especially nuclei of L-/M-cones) was also observed in Nr2e3$^{-/-}$ zebrafish, which could have led to the degeneration. This disorganization seemed to have little effect on UV- or S-cone survival. Analysis of a 77-year-old postmortem ESCS retina showed that the number of S-cones was maintained [30]. Besides, S-cones can survive normally to 5 months in rd7 mouse and 31 weeks in Nrl$^{-/-}$ mouse [24,35]. UV-and S-cones can survive to 10 months in Nr2e3−/− zebrafish. It is possible that the interaction between rods and L-/M-cones is more critical than that with UV- or S-cones.

Our work has extended the knowledge of Nr2e3 and provides interpretation of the phenotypes in ESCS and rd7 mouse. In consideration of the complex phenotypes of ESCS and the rd7 mouse,

causality of the underlying mechanisms that involve multiple cell types is difficult to determine. Thus, it is impossible to identify therapeutic targets to save the cone photoreceptors, which are the key essential cells for useful human vision. By contrast, the Nr2e3-null zebrafish model provides a clear target for developing therapeutic intervention, i.e., survival of the L-/M-cone photoreceptors. This feature, in addition to the intrinsic advantages of the zebrafish model, may offer new opportunities in finding treatments for ESCS in particular and retinal degeneration in general.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 31571303, 81500762, 81670890, 81870691,
31871260 and 81770102), Research Funding for the Doctoral Program of Higher Education of China (20120142110077).

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Figure legends

Figure 1 Generation of Nr2e3 knockout zebrafish. (A) The eight exons of zebrafish Nr2e3 and the genomic target site are shown. The target sequence is highlighted. (B) Sanger sequencing of the target site in wildtype (WT) and Nr2e3−/− zebrafish to confirm the genotype of c.485_521del (del37). The deleted sequence is indicated in the box. (C) Nr2e3 mRNA levels in WT and Nr2e3 mutant were examined by quantitative PCR at 36 hpf. Five independent experiments were performed. β-Actin served as endogenous control. The results are shown as mean ± SD. ***P < 0.001. (D) Nr2e3 protein levels in WT and Nr2e3 mutant were detected at 1 month old larvae by western blotting.

Figure 2 *Nr2e3*$^{-/-}$ zebrafish present a no-rod retina. (A) Retinal cryosections from wildtype (WT) and Nr2e3 mutant were immunostained with anti-rhodopsin antibody at 10 dpf (dorsal is up), 6 months and 10 months (images come from the ventral retina). Confocal images showed there are no rods at all stages in mutant retinas. Scale bars: 20 μm. (B) Quantitative PCR assays at 5 dpf demonstrated rod genes rhodopsin, gnat1, grk1a and pde6b were not expressed in Nr2e3 mutants. Three independent experiments were performed. β-Actin served as endogenous control. The results are shown as mean ± SD. ***P < 0.001. (C) Western blotting analysis at 2 months showed the proteins that participant in the rod phototransduction cascade were not expressed in Nr2e3 mutants. α-Tubulin was used as endogenous control.

Figure 3 Ultrastructural analysis of photoreceptor OSs in wildtype (WT) and Nr2e3−/− retinas. (A) At 10 dpf, recognizable rod OSs developed well in WT retinas, (B) whereas only cone OSs were observed in Nr2e3 mutant retinas. (C) At 1 month of age, well-formed rod OSs were regularly organized in WT retinas; (D) similarly, no rod OSs were observed in Nr2e3 mutant retinas. (C', D') Enlarged regions of the boxes in C and D. Arrowheads indicate the rod OSs, whereas arrows indicate the cone OSs. Scale bars: 1 μm in (A, B); 5 μm in (C, D and C', D').

Figure 4 Generation of rod lineage is not affected in *Nr2e3*$^{-/-}$ zebrafish. (A) Pax6a mRNA levels were examined at 36 hpf and 48 hpf. Three independent experiments were performed. β-Actin served as endogenous control. The results are shown as mean ± SD. NS, no significance. (B) Confocal images of the EdU labelled cells are shown at 60 hpf. Arrowheads indicate the rod progenitors or precursors. (a, b) Magnified images of the boxes in B. (C) Confocal images stained for zpr-3 at 72 hpf. WT, wildtype. Scale bars: 20 μm in (B, C); 10 μm in (a, b).

Figure 5 Development of cone photoreceptors is normal in Nr2e3−/− zebrafish. Confocal images stained for UV opsin (A), red opsin (C) and green opsin (D) at 5 dpf, as well as blue opsin at 7 dpf (B). The number of cone photoreceptors and distribution of cone opsins were normal. WT, wildtype. Scale bars: 20 μm.

Figure 6 Retinal lamination is retained in *Nr2e3*$^{-/-}$ zebrafish despite OSs degeneration. Retinal cryosections from wildtype (WT) and Nr2e3 mutant at 10 dpf (A), 1 month (B), 4 months (C) and 12 months (D) were stained with hematoxylin and eosin. RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 40 μm.

Figure 7 L-/M-cones is degenerated progressively in *Nr2e3*$^{-/-}$ retinas. Confocal images stained for red opsin (A) and green opsin (B) at 1 month, 6 months and 12 months. The length of OSs of these two cones was shortened gradually from 1 month. Scale bars: 20 μm. (C and D) The length of L-/M-cones OSs was statistically analyzed at 1 month, 6 months and 12 months. WT, wildtype. Data are presented as mean ± SD. **P < 0.01, ***P < 0.001.

Figure 8 Nr2e3 enhances rhodopsin gene expression but does not repress cone opsin genes. Reporter plasmids contain the promoter sequence of zebrafish rhodopsin (A), UV-opsin (B) and S-opsin (C) were transfected into HEK293 cells alone or combined with the indicated expression vectors of Nr2e3, Crx and Nrl. The relative activity of each promoter is shown. '+', '++' and '+++' represent 100, 200 and 400 ng of the indicated vector, respectively. To confirm the total amounts of DNA were constant between pores, empty plasmid was added. Three independent experiments were performed. The results are presented as mean ± SD.

Figure 1

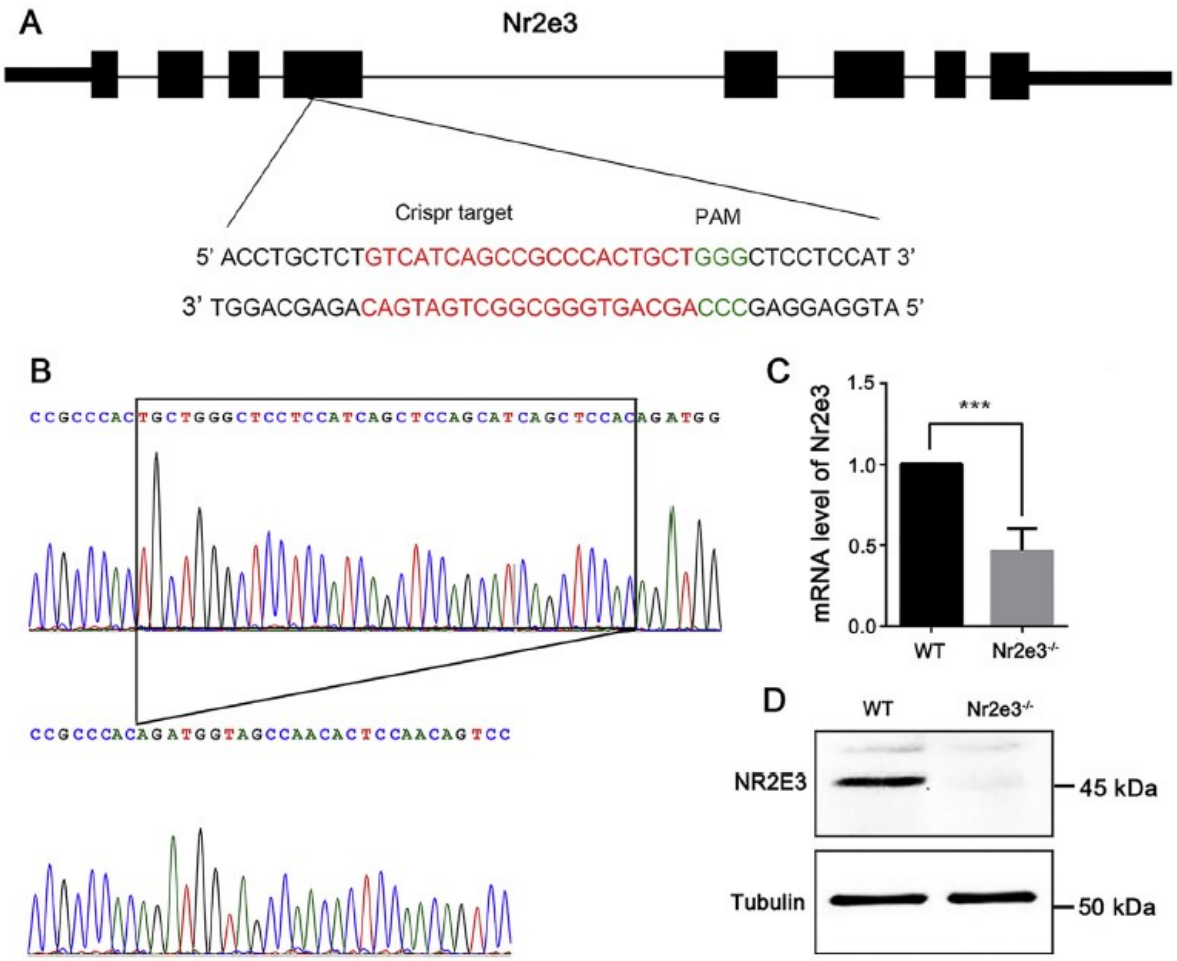

A
Nr2e3
Crispr target
PAM
5' ACCTGCTCTGTCATCAGCCGCCCACTGCTGGGCTCCTCCAT 3'
3' TGGACGAGACAGTAGTCGGCGGGTGACGACCCGAGGAGGTA 5'
B
CCGCCCACTGCTGGGCTCCTCCATCAGCTCCAGCATCAGCTCCACAGATGG
CCGCCCACAGATGGTAGCCAACACTCCAACAGTCC
C
mRNA level of Nr2e3
1.5
1.0
0.5
0.0
***
WT
Nr2e3-/-
D
WT
Nr2e3-/-
NR2E3
45 kDa
Tubulin
50 kDa

Figure 2

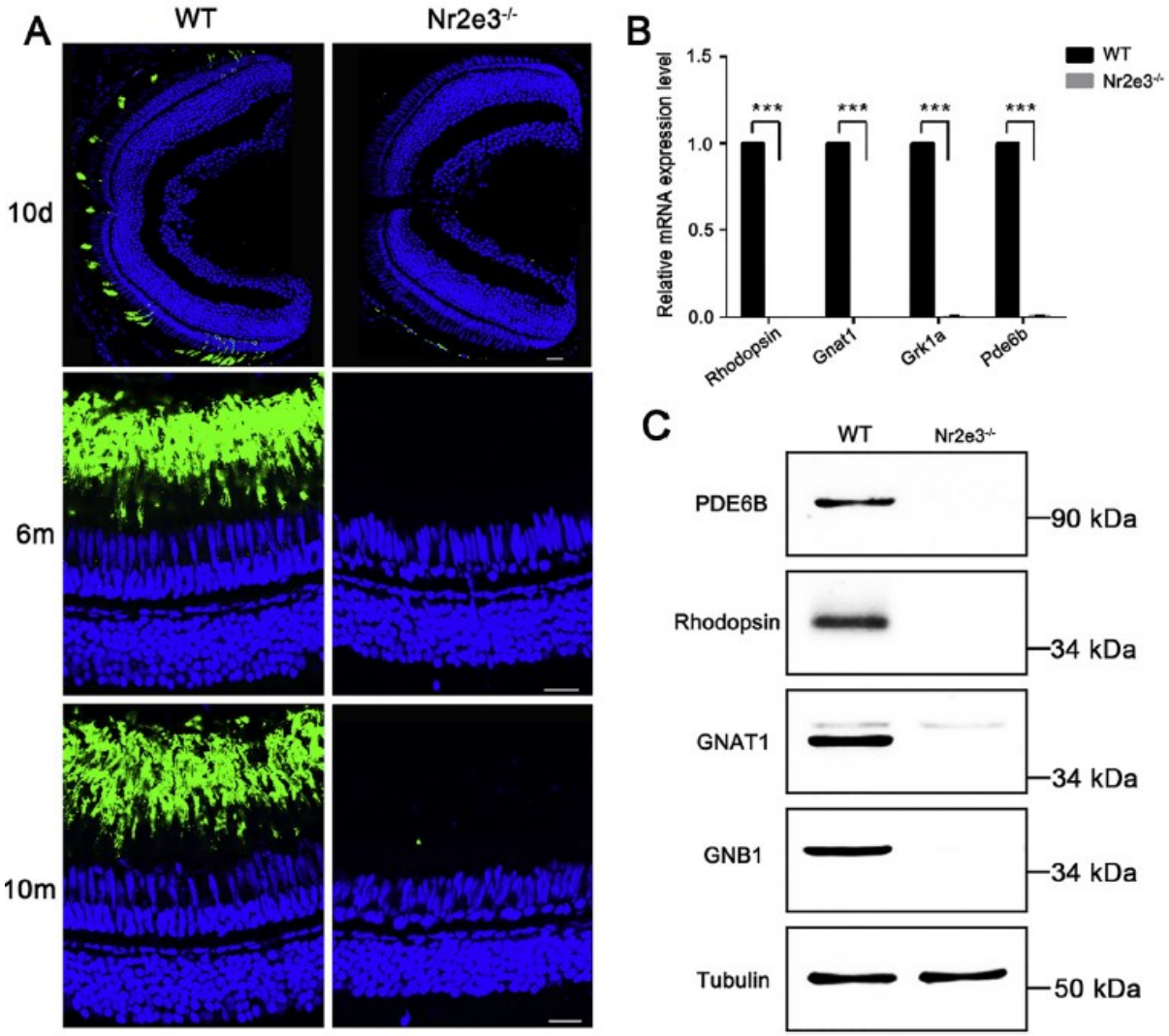

A
WT
Nr2e3-/-
10d
6m
10m
B
Relative mRNA expression level
1.5
1.0
0.5
0.0
WT
Nr2e3-/-
***
***
***
***
Rhodopsin
Gnat1
Grk1a
Pde6b
C
WT
Nr2e3-/-
PDE6B
90 kDa
Rhodopsin
34 kDa
GNAT1
34 kDa
GNB1
34 kDa
Tubulin
50 kDa

Figure 3

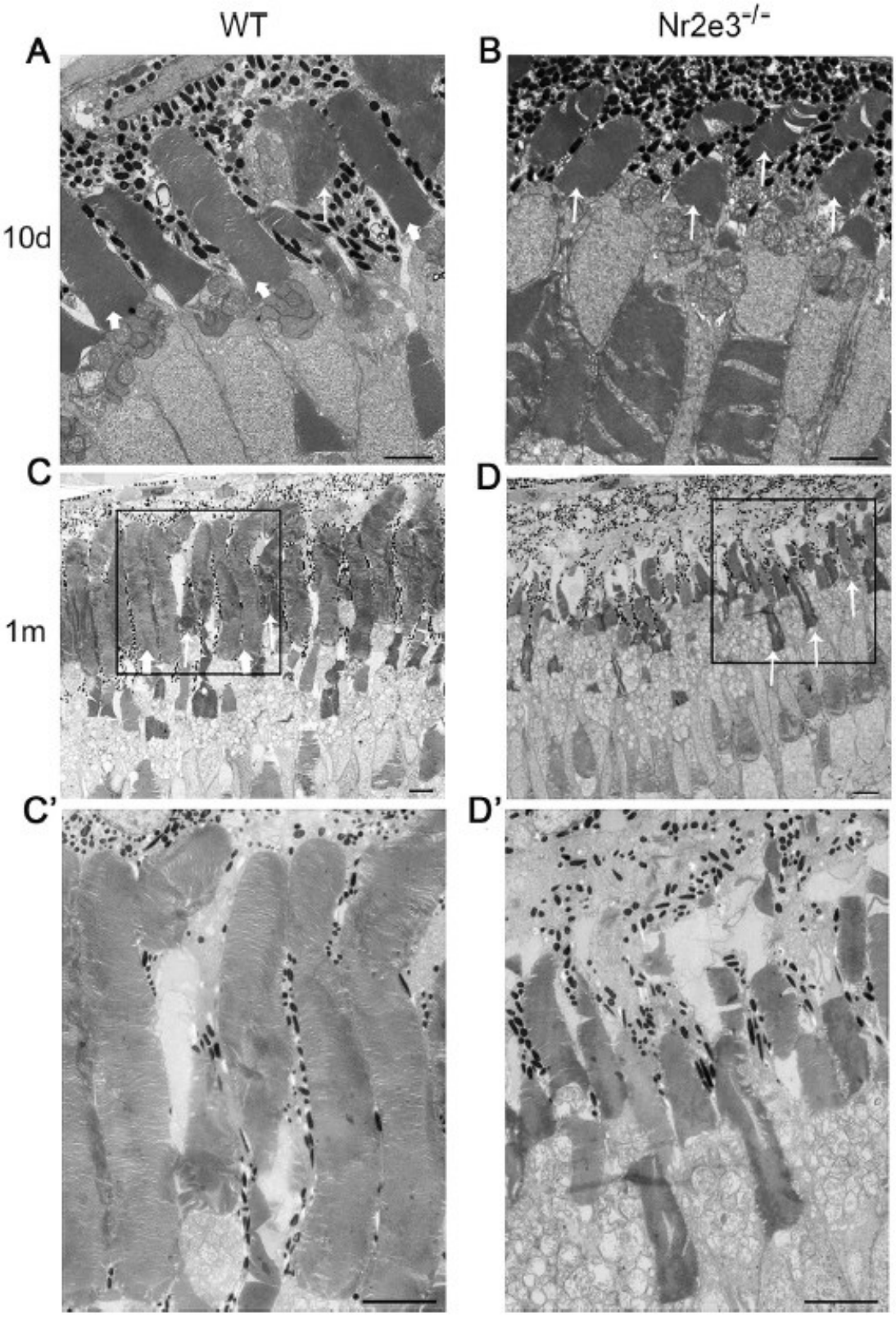


Figure 4

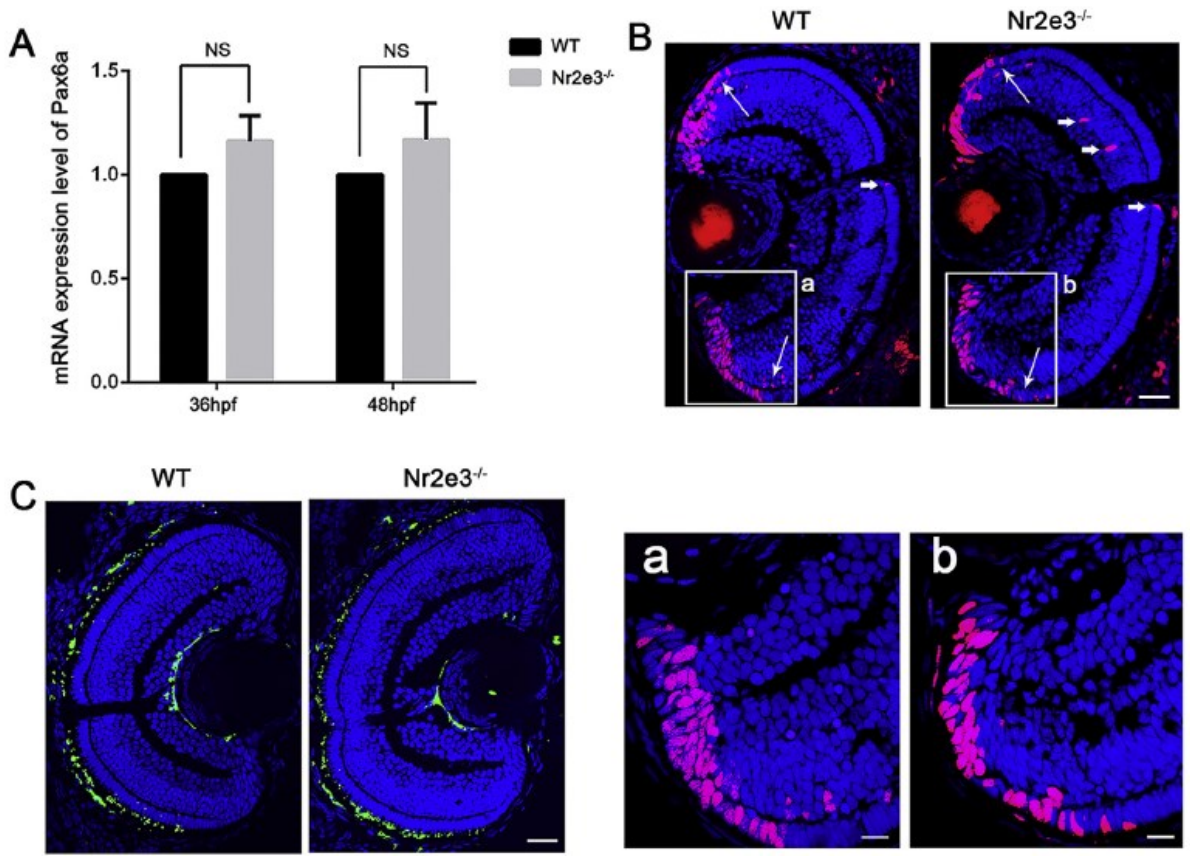

A
mRNA expression level of Pax6a
1.5
1.0
0.5
0.0
NS
NS
36hpf
48hpf
WT
Nr2e3-/-
B
WT
Nr2e3-/-
a
b
C
WT
Nr2e3-/-
a
b

Figure 5

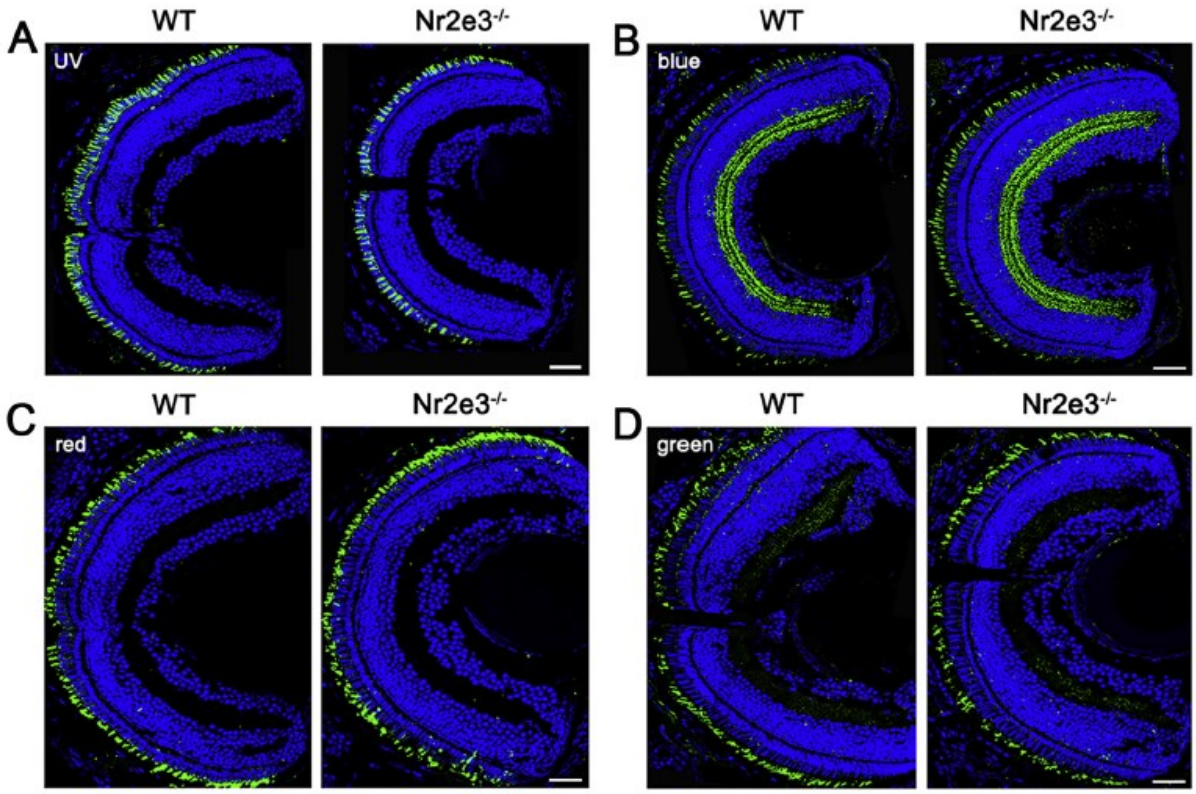


Figure 6

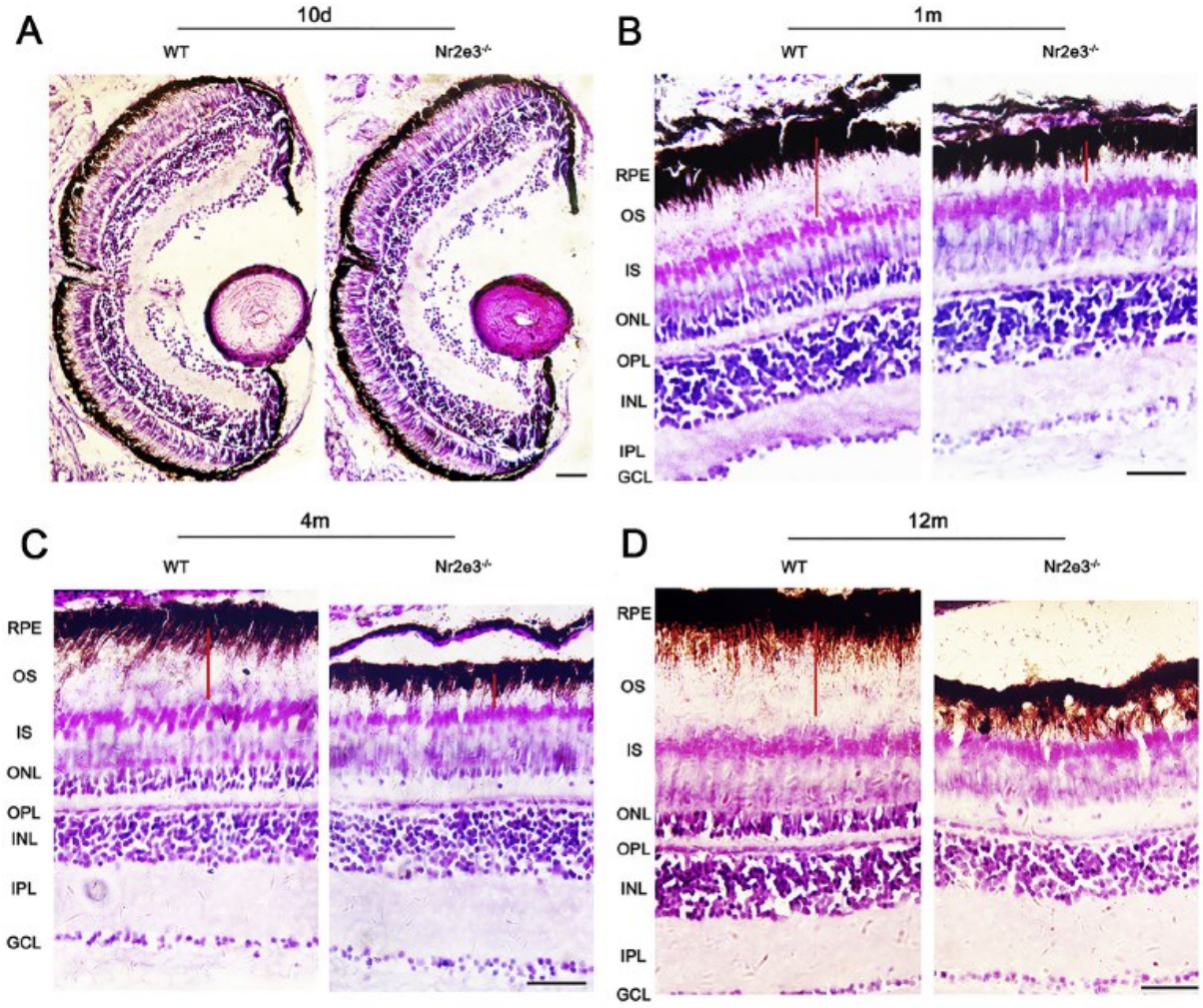

A
10d
WT
Nr2e3-/-
B
1m
WT
Nr2e3-/-
RPE
OS
IS
ONL
OPL
INL
IPL
GCL
C
4m
WT
Nr2e3-/-
RPE
OS
IS
ONL
OPL
INL
IPL
GCL
D
12m
WT
Nr2e3-/-
RPE
OS
IS
ONL
OPL
INL
IPL
GCL

Figure 7

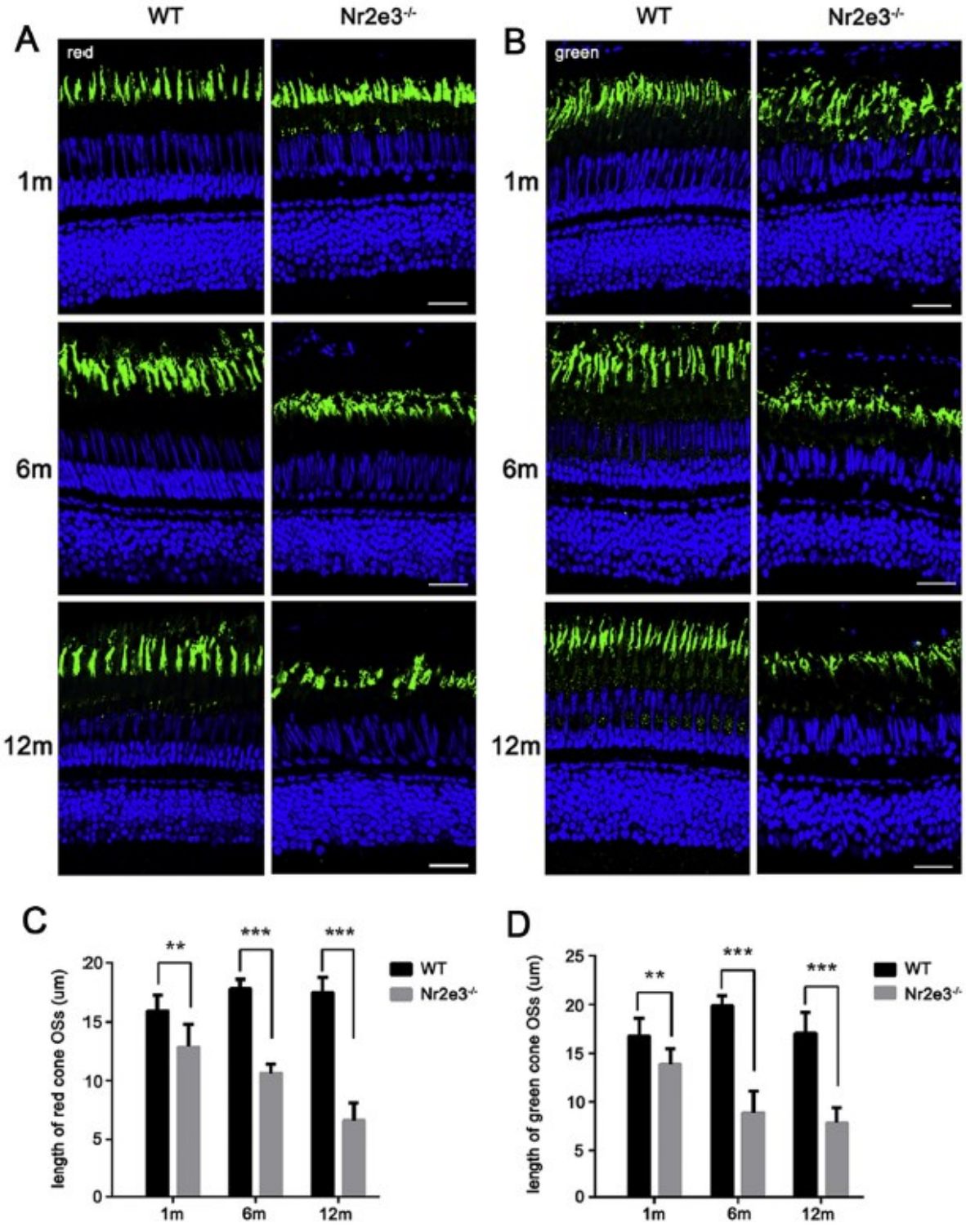
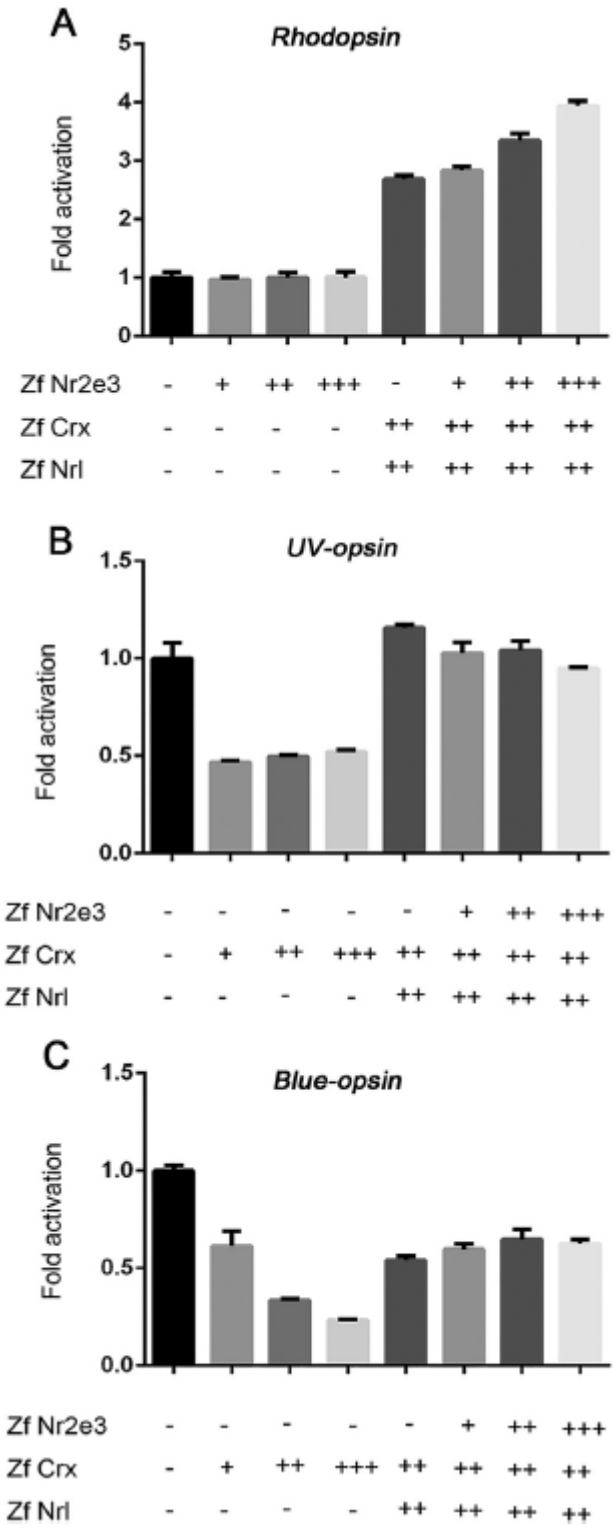

A
WT
Nr2e3-/-
red
1m
6m
12m
B
WT
Nr2e3-/-
green
1m
6m
12m
C
length of red cone OSs (um)
**
***
***
WT
Nr2e3-/-
0
5
10
15
20
1m
6m
12m
D
length of green cone OSs (um)
**
***
***
WT
Nr2e3-/-
0
5
10
15
20
25
1m
6m
12m

Figure 8


A
Rhodopsin
Fold activation
0
1
2
3
4
5
Zf Nr2e3 - + ++ +++ - + ++ +++
Zf Crx - - - - ++ ++ ++ ++
Zf Nrl - - - - ++ ++ ++ ++
B
UV-opsin
Fold activation
0.0
0.5
1.0
1.5
Zf Nr2e3 - - - - - + ++ +++
Zf Crx - + ++ +++ ++ ++ ++ ++
Zf Nrl - - - - ++ ++ ++ ++
C
Blue-opsin
Fold activation
0.0
0.5
1.0
1.5
Zf Nr2e3 - - - - - + ++ +++
Zf Crx - + ++ +++ ++ ++ ++ ++
Zf Nrl - - - - ++ ++ ++ ++